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**EFFECTS OF MICROWAVE RADIATION ON
NEURONAL ACTIVITY**

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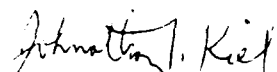
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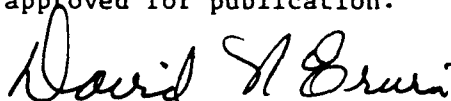
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This report has been reviewed and is approved for publication.



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13. ABSTRACT (Maximum 200 words) A microwave radiation device was designed and constructed for exposure of fetal rat neurons during microscopic observation. The device exposed growing neurons to 400 MHz radiation amplitude modulated at 16 Hz. Continuous exposure to radio-frequency radiation for 4 consecutive days led to the development of a cell number density gradient. The greater number of cells occurred in the center of the culture plate which was directly in the field as opposed to the more peripheral areas of the plate which were outside of the field. Nonirradiated control cultures did not display this gradient. This finding was replicated under various exposure periods. The gradient was formed within 20 min of placing the plates on the antenna.				
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EFFECTS OF MICROWAVE RADIATION ON NEURONAL ACTIVITY

SUMMARY

The effects of radiofrequency radiation on rat hippocampal fetal neurons were examined. Carrier frequencies of 300 to 470 MHz were used, with most experiments carried out at 400 MHz. In some experiments the amplitude of the radiofrequency (RF) carrier was modulated, and the amplitude modulated frequency ranged from 10 Hz to 300 Hz. Neurons exposed to the RF field for any period up to 14 days appeared to be perfectly normal morphologically using phase contrast microscopy. Temperature measurements using a vitek probe revealed that culture dish temperature was maximum directly over the axis of symmetry of the antenna, but never exceeded 3 °C above the ambient temperature. A reliable effect observed in irradiated dishes was a concentration of neurons within the RF field. This density gradient was established within 20 min of placing the dish on the antennae, but did not occur if cells were allowed to attach to the dish before irradiation. The concentration phenomenon was not specific to neurons and was observed in both HeLa and Chinese Hamster Ovary (CHO) cell cultures. The evidence does not support changes in division rate or increased survival rate as explanations of the density gradient. A likely mechanism is that thermal convection currents within the irradiated dishes increase the probability of cells settling and attaching to the dish surface directly above the antenna. This technique is currently being exploited as a means of concentrating neurons within a small area to facilitate investigation of neural networks.

INTRODUCTION

The medical uses of radiofrequency (RF) and microwave (MW) radiation, encompassing electromagnetic waves (EW) in the frequency range of 10 kHz to 300 GHz, include deep heating of muscles and cancer hyperthermia therapy (30). The possible biological effects of radiofrequency radiation (RFR) at field strengths that produce minimal, or no, thermal changes has been addressed by recent articles in popular magazines, hence public interest has been piqued about the weak electric and magnetic fields that are ubiquitous in modern industrial environments. In particular, studies linking leukemia in children and residential proximity to power transformers (29), and reports of increased incidence of leukemia among electrical workers (22) have raised concerns. Our purpose here is not to resolve all of the questions that have been raised concerning the reliability of the health effects data, but rather to use current in vitro RFR exposure techniques on cultured fetal hippocampal neurons to determine the response of these extremely active, developing

cells to electromagnetic fields. We recognized that mechanisms responsible for athermal biological responses have not been unequivocally defined, but data in the literature on neurite growth patterns (20,25-27), increased protein synthesis (16,17), and ion fluxes (3,4,6,7) in electromagnetic fields (EMF) provide compelling evidence that further research will elucidate important mechanisms concerning how electromagnetic signals interact with cells.

It has been shown that exposure of diptera salivary glands and human cultured cells to extremely low-frequency (ELF) EMF alters patterns of polypeptide synthesis (18,19). The effects displayed signal waveform dependence and only slight overlap with polypeptide synthesis produced by heat-shock conditions. Since increased neuronal expression of c-Fos immunoreactivity occurs after stress (9), a similar increase in immunoreactivity in neurons exposed to RFR would signify that the fields are producing a perturbation in the cells' environment that is being detected and transduced to the nucleus. Elevated intracellular calcium concentration is one mechanism of c-Fos stimulation in neurons and should be investigated if RFR induces protein expression.

Several laboratories have focused on ionic fluctuations observed in the presence of ELF modulated RFR EMF. Increased efflux of $^{45}\text{Ca}^{2+}$ from human neuroblastoma cells (IMR 32) occurred during exposure to 147-MHz RFR that was amplitude modulated at ELF of 13, 16, 57.5 and 60 Hz (14). The effects were not linearly related to power density, in that significant changes occurred only at specific absorption rates (SAR) of 0.005 and 0.05 (W/kg). These results agree with similar cell culture studies using 915-MHz RFR (13) and experiments using preparations of fresh chick and cat brain tissue (3,4,6). The reported effects of RFR on calcium and other ions are varied and include both increased release, as just discussed, and increased uptake (15). Increased norepinephrine release has also been observed (12). In reviewing these results one is prompted to ask why only certain ELF modulations and "windows" of power density produce effects. Blackman et al. (8) discuss possible answers in terms of energy amplification and cooperativity processes that are set in motion so that membrane parameters, such as microviscosity, suddenly become extremely sensitive to small EMF disturbances. The entire issue of how very small energy levels of athermal RFR can significantly alter biological functions, when current intensities generated by cells are orders of magnitude larger, is one that has frequently been raised and has been addressed in depth by Adey (1). Several theoretical models have been proposed to explain the observed phenomena (1,7,8).

Other effects that have been observed when cells are exposed to electric fields, in this case steady, uniform ones, include changes in alignment of fibroblasts (27) and accelerated neurite growth toward the cathode, or negative electrode (25).

These responses are thought to be related to migration of membrane proteins, which has been demonstrated in muscle cells (21,24). We do not know if neurite alignment or synaptic densities are altered for neurons located inside, outside, and at the edge of an EMF.

METHODS AND PROCEDURES

Experimental Animals

One pregnant female Sprague Dawley rat, purchased from Harlan Sprague Dawley, was delivered every 2 weeks so that arrival date coincides with gestation day 15. The animal was housed in Isocages (Lab Products, Inc.) with white pine shaving litter at the temperature and humidity controlled University Animal Life Facility. A 12 h light/dark schedule was maintained, and food (Wayne Lab Blocks) and water were available ad libitum.

Cell Culture Preparation

Hippocampal neurons were prepared from 19-day-old fetuses in a procedure similar to that described by Bakker and Cowan (2). The dam was anesthetized with Nembutal (60 mg/kg) and the fetuses removed and placed in chilled, sterile (4 °C) buffered saline. Hippocampi were removed and incubated for 15 min at room temperature in Hank's balanced salt solution (Ca^{++} , Mg^{++} -free) containing 0.1% trypsin. The hippocampi were then passed 10-15 times through a small bore, fire-polished Pasteur pipette, and the resulting dissociated cells collected by centrifugation and resuspended in Dulbecco's modified Eagle's medium (DMEM) (GIBCO No. 430-1600 EB, NaHCO_3 -free) which contained 25-mM Hepes (ph 7.4), 10% fetal calf serum, 10% heat-inactivated horse serum, 100 ug/ml streptomycin, 100 units/ml penicillin G, 10 ug/ml gentamicin sulfate, and 0.25 ug/ml amphotericin B. The cells were added to 35-mm Corning tissue culture dishes (at a density of 1 fetal hippocampus per 2 dishes) that were treated for 2 h with 0.1 mg/ml poly-L-lysine hydrobromide (30-70 kd, Sigma Cell Culture Reagent) in water. After 2 h at 37 °C, the medium was replaced with one containing N-2 supplements (insulin, transferrin, putrecine, progesterone, sodium selenite). The cells were equilibrated with air rather than 5-10% CO_2 and were placed in bicarbonate-free medium to repress the division of glia and allow easier observation of neurons. Using these procedures neurons have been cultured for up to 30 days with a change of one-half of the medium every 7 days.

Irradiation Procedures

The culture dish containing neurons were placed on the brass plate antenna which is attached to a 50-ohm coaxial cable

so that the center conductor is flush with the brass plate and the bottom center of the dish. The center conductor diameter is 1.8 mm and the inner diameter of the outer conductor is 5.5 mm. The RF carrier frequency was provided by an HP 8644A signal generator. The carrier was amplitude modulated by mixing with either an HP 8644A internal wave or with a low frequency signal from an HP function generator. A linear microwave power amplifier (Airep Electronics) amplified the signal. Transmitted and reflected powers were measured via a dual-directional coupler (HP 778D) connected to a dual-sensor power meter (HP 438A), which coupled directly to the output of the Airep amplifier (13). The irradiation was conducted within the incubator. Control data was collected from 2 sources: (1) neurons in the same dish that are outside of the center electric field, and (2) neurons from dishes that receive identical treatment except for the presence of RFR.

A vitek 4-conductor probe was used to measure the temperature of the irradiated medium. The vitek probe has 2 leads for powering the device and 2 leads (output leads) for observing the voltage across the thermistor that is incorporated into the tip of the device. In different experiments the vitek probe was powered by either a 3-v battery or 5-v/1,000-Hz sinusoidal voltage applied across its 2 power leads. The voltage across the temperature sensitive resistor in the probe was measured by monitoring the potential across the 2 output leads. These high resistance leads were connected to an ultra-high input impedance quad JFET integrated circuit (IC) amplifier (LF347), which was used to buffer the signal. The 2 buffered signals were then fed into the inverting/noninverting inputs of a high precision instrumentation amplifier IC (AD624) in order to take the difference of the 2 inputs and amplify the resultant. In the case of the sinusoidally driven probe, the signal was synchronously demodulated using an Analog Devices 630 IC and the output was low-pass filtered to extract the 1,000-Hz component of the original unmodulated signal. The advantage of this approach is that thermal DC drifts in the electronics, 1/f noise and the line noise pickup are filtered out. The processed voltage was fed into an AD389 sample and hold amplifier, which was then digitized using an ADC72 16-bit analog-to-digital converter. An IBM PC interface was constructed (with DMA capability). Software was written (versions in assembler, FORTH and C) to program the IBM PC interface and collect the temperature-dependent voltage data. Data reduction programs were written in C language to extract temperature from voltage data. The 16-bit temperature data were collected, analyzed, and stored on a hard disk.

A precision temperature reference was not available for calibration of the probe to a $\pm 0.02^{\circ}\text{C}$ standard. Using less accurate equipment, the probe was calibrated to a $\pm 0.3^{\circ}\text{C}$ standard. Future accuracy will easily be increased by at least an order of magnitude with the addition of a precision temperature reference to the laboratory.

The carrier frequencies used were in the high RF range and varied from 300 MHz to 470 MHz, with most experiments carried out at 400 MHz. In some experiments the amplitude of the high RF carrier was modulated, and the amplitude-modulated frequency ranged from 10 Hz to 300 Hz. Most experiments were carried out with no modulation of amplitude. During irradiation, transmitted and reflected powers were measured with an HP 438A power meter. This instrument has a GPIB (=HP-IB) computer interface. The power meter was connected to an IBM RT, also equipped with a GPIB interface. A C program was written to periodically poll the power meter and obtain transmitted and reflected power measurements as a function of time. The computer signaled an alarm in the event of a reduction or failure of transmitted power.

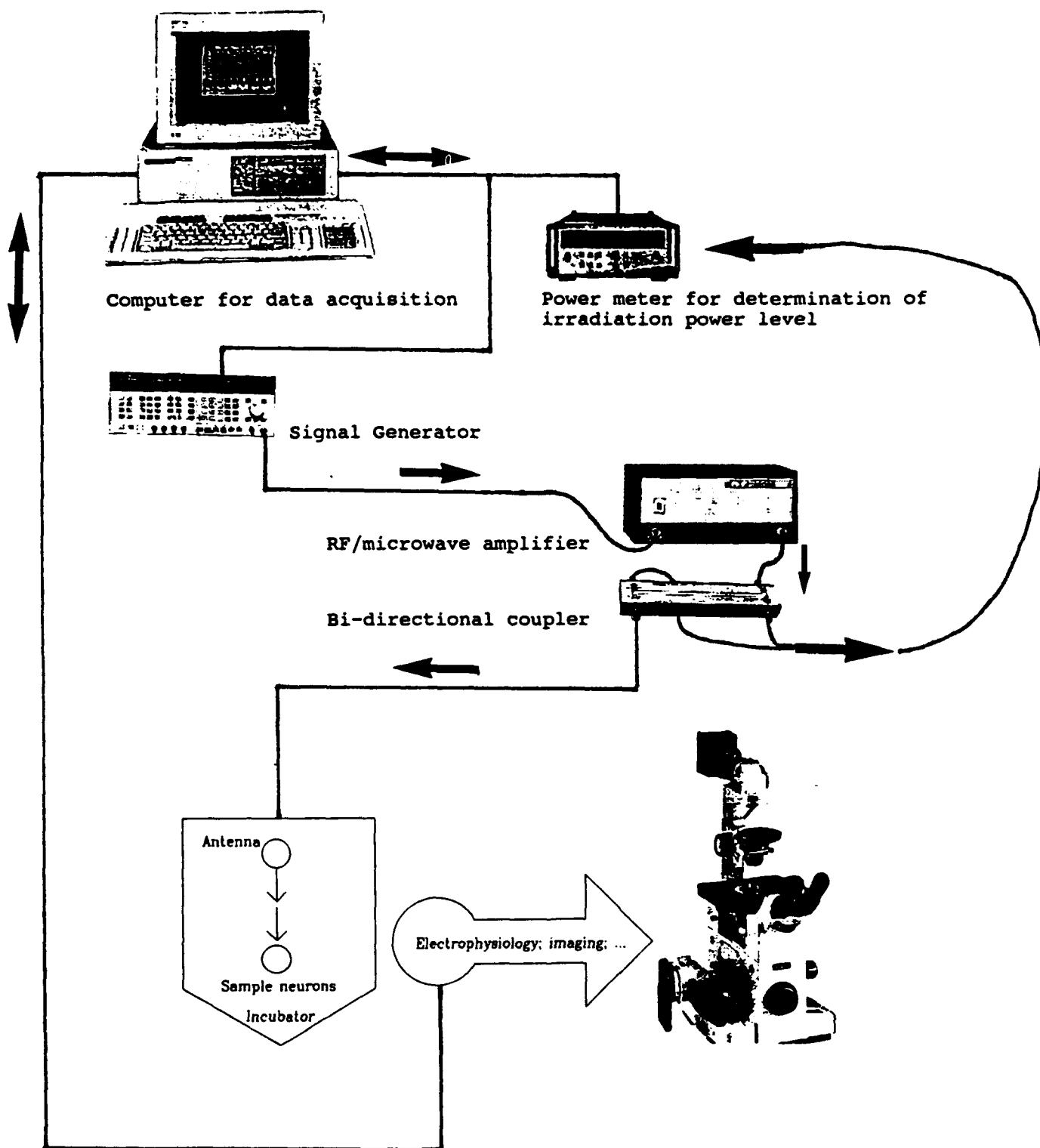
RESULTS

Irradiation System

The following instruments were integrated into an experimental system for irradiating hippocampal neurons in culture: HP 8644A signal generator, HP 438A dual-sensor power meter with sensors, HP 778D microwave dual-directional coupler, Airep Electronics microwave power amplifier, and HP 54502A digitizing oscilloscope (Fig. 1). The antenna is based on the design of Seaman, Burdette, and Dehaan (28) consisting of a brass plate attached to a coaxial cable so that the center conductor is flush with the brass surface which provides support for the culture dishes. A nonperturbing Vitek temperature probe was interfaced with an IBM PC to allow for mapping of temperature gradients in and around the electric field.

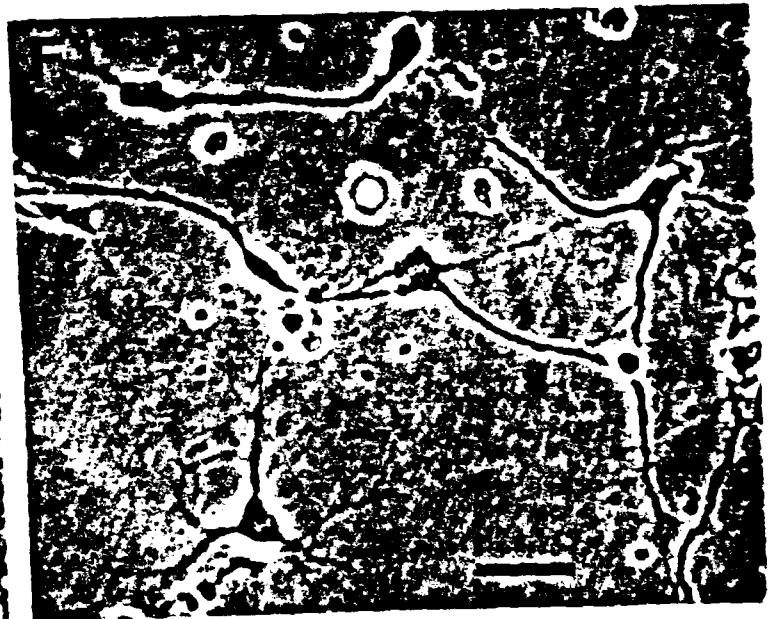
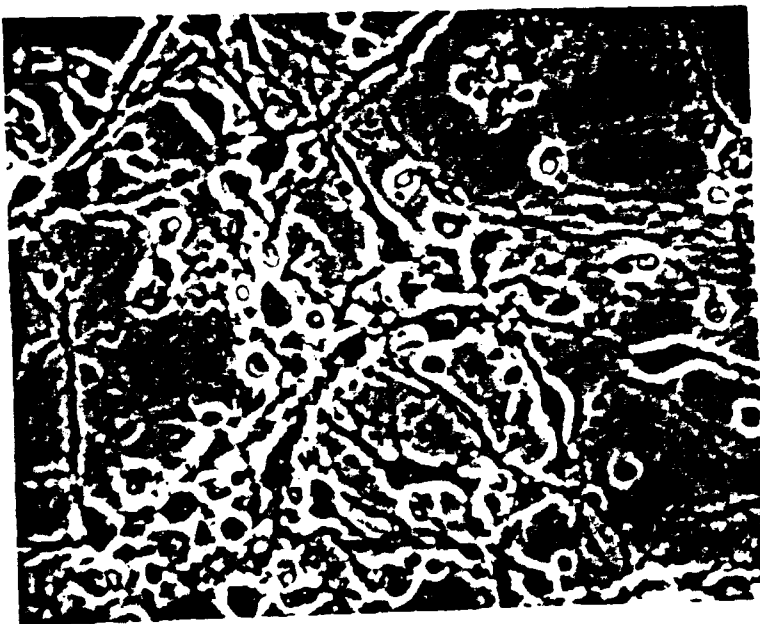
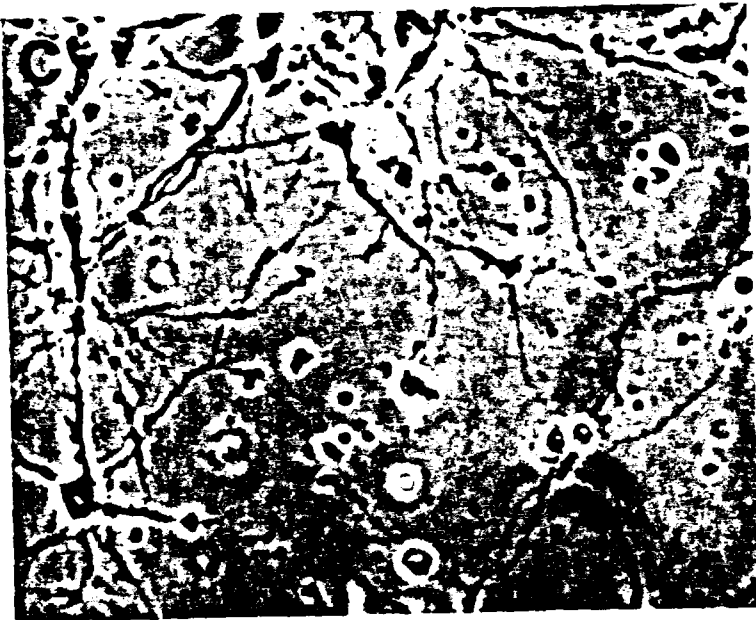
Temperature Changes During Irradiation

As might be expected, we found that the temperature of an irradiated sample was maximum at the center of the dish, directly on the axis of symmetry of the antenna, and fell off steeply to the background temperature as one moved off of the symmetry axis by one mm. The maximum never exceeded 3 °C above the ambient temperature. This temperature change was independent of the carrier frequency and/or modulation used in these experiments.



(Photo on Facing Page)

Figure 1. Diagram of irradiation equipment and integration with cell culture incubator. The microscope rests on an air table and is equipped with micromanipulators, a 35-mm camera, and video camera port.



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Figure 2. Effects of temperature and radiofrequency radiation on neuronal morphology. Cells were either placed in a 37 °C incubator, a 40 °C incubator, or on the RF antenna in a 37 °C incubator from the time of plating through day 5. Photographs were then taken of cells using a phase contrast microscope at either center or periphery of the culture dish. A) 37 °C center, B) 37 °C periphery, C) 40 °C center, D) 40 °C periphery, E) RF center, and F) RF periphery.

Characteristics of Cultured Neurons

The rat fetal hippocampus contains a total of 900,000 pyramidal neurons (2). Since we placed the hippocampal tissue from 1 fetus on 2 plates, we applied 450,000 pyramidal cells to each 35-mm culture dish. Our results agree with those of Banker & Cowan (2) in that 90% of the applied pyramidal cells attach to the dish. These neurons are derived mainly from areas CA1 and CA3 of the fetal hippocampus. Dentate gyrus granule cells are smaller (soma diameter = 10 μ m) and do not attach to the polylysine-coated substratum. These cells remain in suspension and do not extend neurites in our culture system. The detectable glia are flat, polygonal Type I and star-shaped, Type II astrocytes. These cells stain positively with antibody to the astrocyte marker protein, glial fibrillary acidic protein (GFAP). Other glial cells (oligodendrocytes, ependymal cells, microglia) do not survive under our culture conditions. The pyramidal cells are positively stained with antibody to the neuronal marker microtubule-associated protein 5 (MAP5) which is also called MAP1B. The pyramidal cells begin to extend neurites as soon as the cells attach to the substratum, which occurs within 30 minutes after application to the dishes.

Neuronal Responses to Irradiation

In preliminary experiments several dishes of freshly plated hippocampal neurons were placed on the larger brass antenna and irradiated for 4 h every 24 h for 3 to 4 days. The carrier signal frequency was 400 MHz with an amplitude modulation of 16 Hz. Morphological examination revealed that cell viability was reduced in both irradiated and nonirradiated control plates. This effect was attributed to poor temperature regulation of the plates while they are removed from the incubator and placed on the antenna. Subsequently all cells were irradiated within the incubator itself.

Culture dishes continuously exposed to the RF field for 4 consecutive days develop density gradients, with a greater number of cells occurring in the center of the plate which is directly in the field as opposed to the more peripheral areas of the plate which are outside of the field. Nonirradiated control plates do not display this density gradient (Fig. 2). This finding has been replicated using exposure periods ranging from 15 min to 14 days, with the plates being removed only briefly from the antenna for medium changing every 7 days. Durations longer than 14 days were not investigated because the number of neurons per dish declines normally with time in culture, and after 2 weeks about 25% of the cells originally plated are still present.

The density gradient is established within 20 min of placing the plates on the antennae. The concentration phenomenon is not neuron specific since similar density gradients are observed when both HeLa cells and Chinese Hamster Ovary (CHO) cells are plated and exposed to the RF field for 20 min. Neurons exposed to the field for any period up to 14 days appear to be entirely normal morphologically using phase contrast microscopy. In fact, there appears to be even a greater density of neurites in the RF-exposed cells, and this may be a function of the higher temperature in the center of the dish.

DISCUSSION

Cultured fetal hippocampal neurons were not adversely affected by continuous exposure to RF radiation in the 300 MHz to 470 MHz range. Although lacking a precise temperature reference for calibrating the vitek probe posed some difficulty, measurements consistently revealed that a 3 °C increase occurred in the culture dish directly on the axis of symmetry of the antennae. Temperature controls conducted in an incubator set at 40 °C revealed that the fetal neurons survive and develop neurites quite well at this temperature, but that neurons and glia are both killed within 24 hours if they are kept at 42.4 °C. These findings confirm that temperatures produced in the center of the RF field must be lower than 42 °C. The most striking phenomenon was an obvious increase in the number of neurons within the RF field as opposed to the periphery of the culture dish. It was very exciting to think that perhaps the few neurons that were still dividing when plating occurred were somehow stimulated to continue division. However, this does not appear to be the case. The fact that the concentration phenomenon occurred within 20 min of plating the cells indicates that the increase in cell number is not due to cell division since this period of time is too short for mitosis to occur. In experiments using continuously dividing cells such as HeLa and CHO cells, the same rate of division occurred in RF-exposed and control plates. In all cases the density gradient of cells did not occur if cells were allowed to fully attach to the dish before placement on the antenna. These results indicate that the increase in cell number at the center of RF-exposed dishes results from convection currents set up by a thermal gradient in which the temperature was higher in the center of the dish than at the periphery. Thus single cells floating in the medium might be drawn up by these currents and then have a greater probability of settling and attaching above the antenna. Although this finding is not particularly exciting, it does provide a means of concentrating healthy neurons within a small area, which facilitates optical recording of neuronal networks. We are currently working on exploiting this technique.

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